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MicroRNAs in the Ionizing Radiation Response and in Radiotherapy

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Abstract

Radiotherapy is a form of cancer treatment that utilizes the ability of ionizing radiation to induce cell inactivation and cell death, generally via inflicting DNA double-strand breaks. However, different tumors and their normal surrounding tissues are not equally sensitive to radiation, posing a major challenge in the field: to seek out factors that influence radiosensitivity. In this review, we summarize the evidence for microRNA (miRNA) involvement in the radioresponse and discuss their potential as radiosensitizers. MicroRNAs are endogenous small, noncoding RNAs that regulate gene expression post-transcriptionally, influencing many processes including, as highlighted here, cellular sensitivity to radiation. Profiling studies demonstrate that miRNA expression levels change in response to radiation, while certain miRNAs, when overexpressed or knocked down, alter radiosensitivity. Finally, we discuss specific miRNA-target pairs that affect response to radiation and DNA damage as good potential targets for modulating radioresponsitivity.

Introduction

Radiotherapy is one of the main modalities of cancer treatment. Ionizing radiation (IR) damages cells by producing intermediate ions and free radicals that cause DNA double-strand breaks (DSBs), the most common injury from IR. Failure to repair this type of damage leads directly or indirectly to cell death [1–3]. While some cells undergo apoptosis immediately after irradiation, it is generally thought that tumor eradication occurs by mitotic (or “reproductive” or “delayed”) cell death resulting from IR-induced injury that causes cell inactivation and becomes lethal after a few cell divisions [1,3]. In this case, the most relevant processes to cellular radiosensitivity are the double-strand break repair pathways: homologous recombination (HR) and nonhomologous end-joining (NHEJ) [1,2]. While the DNA damage response pathways play an important role in determining cellular radiosensitivity, other cell survival, cell cycle checkpoint, and cell death pathways are also involved – some of which will be discussed later in this review in the section pertaining to specific miRNAs and their targets.

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Scientists have studied how cells are affected by IR not only to *understand* the effects on cells and organisms, but also as a basis for determining appropriate doses and dose distribution for better therapeutic outcome [1]. Moreover, we can further avail ourselves of these insights to develop radiosensitizing agents. While we know of many protein-coding genes that function in response to IR, it is not always easy to manipulate those genes directly. This is where microRNAs (miRNAs) as inherent gene regulators could offer new possibilities.

MiRNAs are a class of endogenous small, non-coding RNAs (~22 nt) with a role in gene expression regulation at the post-transcriptional level. MiRNAs bind to the 3' untranslated regions (UTRs) of their target mRNAs with imperfect base complementarity and repress translation or stability of target mRNAs. Translational inhibition is often a consequence of mRNA deadenylation and degradation [4,5]. The intrinsic ability of miRNAs to act as negative gene regulators allows them to influence signaling pathways that could alter multiple cellular processes, including the response to IR. Therefore, they have the potential to be useful in manipulating the radiation response in the clinic to enhance susceptibility to or protect cells from radiation. While there are currently no miRNAs approved as drugs, much progress is being made in developing them for use as therapeutics [6,7]. Several studies of miRNA delivery, not dissimilar to that of siRNAs, focus on employing modified nucleic acids (for example, locked nucleic acid (LNA) or peptide nucleic acid (PNA)), or packaging those RNAs in nanoparticles [7,8]. LNAs-based miR-122 antagonist is leading the way and is now in phase II clinical trial [7]. Nanoparticle-coated RNAs have also been shown to be deliverable both topically and systemically (intravenously) in mouse models [8–10]. These nanoparticles are usually modified from biodegradable, FDA-approved polymers [8–10], and hopefully would prove possible to move to clinical trials. Delivery of RNAs has met with several challenges in the past and different organs could require different techniques of delivery, but these innovations in the field are emerging as potential solutions.

In this review, we will summarize recent literature on miRNAs that are affected or play a role in the response to damage by photon (x-ray and γ-ray) ionizing radiation.

miRNA profiling in the ionizing radiation response

Ionizing radiation poses danger to genomic integrity, and thus cells have evolved a complex network of pathways to defend themselves [2]. With their inherent roles as gene regulators, it was reasonable to hypothesize that some miRNAs were part of this response network, as was later confirmed (see below). However, the roles of specific miRNAs in this process have only recently begun to be elucidated.

One approach in determining which miRNAs play a role in response to IR is to ask which miRNAs are regulated in response to radiation injury. Many research groups have chosen high-throughput methods, such as high-throughput sequencing and microarray analysis, to associate specific miRNAs with cellular responses to IR. These studies revealed that the expression levels of several miRNAs change significantly upon irradiation, doing so reproducibly across various cell types and at varying dosages of X-ray or γ-ray irradiation. These miRNAs are shown and referenced in Table 1, and we will further discuss several miRNAs that have established functions, especially in cell cycle and apoptosis regulation.

The ***let-7*** family of miRNAs has a role in cell proliferation, cell differentiation, and also acts as a tumor suppressor [11,12]. One of the well-established targets of *let*-7 is *KRAS*, which is part of the MAPK signaling pathway [13]. Increased RAS signaling has been shown to protect cells from IR and promote growth (reviewed in [14]), therefore drawing attention to

the possible role of *let-7* in the radiation response. The expression levels of the *let-7* family of miRNAs have been shown by several miRNA profiling studies to be significantly altered by IR [15–26]. However, there are inconsistencies among various cell lines as to whether *let-7* miRNAs are upregulated or downregulated upon irradiation. For example, all but one of the *let-7* miRNAs (*let-7a* to *i*) were shown to be downregulated in a human lung cancer cell line, a normal lung epithelial cell line [26], and a human artificial tissue system [18]. On the other hand, all members of the *let-7* family were upregulated in irradiated human glioma cell line [22]. While these studies seem to suggest that *let-7* expression is regulated by radiation, the direction of the change in *let-7* expression appears to depend on additional sources, from which we may hypothesize that it is due to *let-7* function or regulation, such as RAS status or the level of LIN-28, which regulates *let-7* [27].

One answer comes from a study describing the mechanism by which *let-7a* and *b* are transcriptionally repressed by p53 following irradiation, and that this mechanism not only depends on functional p53, but also on IR-activated ATM signaling upstream of p53 [28]. In mice with wildtype p53, the decrease in *let-7a/b* levels occurs only in the more radiosensitive tissues (such as bone marrows and lungs) which already express *let-7a/b* at relatively higher levels, and not in the more radioresistant tissues (such as brains and muscles) which normally have lower levels of *let-7a/b* [28]. Further studies are needed to determine if this is the main reason for the disagreement between current profiling studies or if other mechanisms are also involved. More interestingly, further studies should also focus on the potential of using *let-7* as therapeutics in cells with intact p53 functions.

The p53 tumor suppressor is involved in the regulation of cell cycle checkpoints and apoptosis, and thus is part of the DNA damage response and can influence radiosensitivity [29]. One of the direct targets of p53 is **miR-34**, and it has been shown that miR-34 plays a role in cell cycle arrest, proliferation inhibition, and apoptosis (reviewed in [30]). After radiation injury, several miRNA profiling studies found that miR-34 family miRNAs are upregulated in different types of human cell lines [19,20,31] and in the nematode *Caenorhabditis elegans* model system [32]. Apart from miR-34, another miRNA that is well established to target many genes in the apoptotic pathway is **miR-21** (reviewed in [33]), which is consistently upregulated upon irradiation in a variety of normal and cancer cell lines [17,21–23,25,34–36].

Our extensive knowledge of *let-7*, miR-34, and miR-21 and their verified targets and several further studies on their effects on radiosensitivity confirm their roles in IR response [16,25,32,37–40]. However, there are thousands of other miRNAs [41], and our knowledge of the majority of them is limited. Some miRNAs seem to respond to IR only in a cell-specific manner. Yet many of them reproducibly appear to be regulated by IR across cell types and doses (Table 1), possibly highlighting the most interesting candidates involved in IR response. These include the miRNAs in the miR-15a/16-1 cluster, which are upregulated upon IR (Table 1) and are known as tumor suppressors in various tumors, especially chronic lymphocytic leukemia [42]; or the miRNAs in the miR-17-92 cluster, the oncogenic miRNAs which are also involved in the normal development of various tissues [43].

miRNAs that affect cellular radiosensitivity

While it is known that the levels of expression of many miRNAs are affected by irradiation, this does not alone show a causal role in the radioresponse. The obvious next question is whether they actively influence how cells deal with radiation injury. Two studies looked at miRNAs' role on a global level by knocking down key components of the miRNA processing and silencing machinery: DROSHA, DICER, and AGO2 [44,45]. After γ-radiation, endothelial cells have increased rates of apoptosis, and knockdown of either

DICER or AGO2 sensitizes them to IR [44]. However, knockdown of all three proteins individually did not change the number of apoptotic cells in non-small cell lung cancer cell lines [45]. This difference could possibly be due to cell types, the degree of knockdown by siRNAs, or the doses of IR used, for instance, that differ between the two studies [44,45].

On the other hand, a recent study demonstrated that knockdown of DICER or DROSHA leads to decrease in number of cells with foci associated with DNA damage response (DDR) at the damage site, but that decrease depends on damage-site-specific sequence of RNAs processed by DICER and DROSHA and not miRNAs [46]. This study did not address cell survival or its proliferation capability, which were the assays employed by the other two studies to determine cellular radiosensitivity [44–46].

Specific miRNAs that sensitize or protect cells to x-rays or γ-rays are shown in Table 2. Several of them (Table 2, those marked with asterisk) behave in such a way that if their levels of expression decrease in response to IR, then overexpressing these miRNAs makes cells more sensitive to radiation, and vice versa. An excellent example of this is *let-7b*, which is markedly downregulated following IR; and *let-7b* ectopic expression increases radiosensitivity while its inhibitor reduces it [26].

Even without understanding the underlying mechanism of how miRNAs in Table 2 affect sensitivity to radiation, these miRNAs show that cellular radiosensitivity can indeed be manipulated by miRNAs. Still, it is of great importance to understand its molecular basis. In the following section, we will discuss specific examples of radiation response pathways in which miRNAs are known to act.

miRNAs that mediate radiation response in specific pathways

ATM: an early responder to DNA double-strand break

Ataxia-telangiectasia mutated (ATM) kinase is a key signaling gene in the double-strand break-induced DNA damage response. ATM is a Ser/Thr kinase that phosphorylates over 700 proteins in order to orchestrate cell cycle checkpoint activity and DNA repair [47]. One ATM target is the KH-type splicing regulatory protein (KSRP), which regulates the biogenesis of a group of miRNAs at the primary miRNA processing level. DSB-induced, ATM-dependent phosphorylation of KSRP enhances processing of many miRNAs, including miR-21, which is consistently upregulated after IR [48].

On the other hand, there is much interest in identifying regulators of ATM expression, so it is of no surprise that ATM's 3' UTR has been analyzed for miRNA complementary sites. ATM has been demonstrated as a direct target of miR-421 [49] and miR-101 [50], and individual overexpression of these miRNAs downregulate ATM expression and sensitize various cell lines to IR [49–52]. miR-421 has an N-myc binding site in its promoter region; and overexpression of N-myc leads to increased miR-421 level as well as a decrease in the level of ATM in a miR-421-dependent fashion [49].

Histone modification and chromatin remodeling required in DDR

In order to allow DNA repair to occur, the repair proteins need to not only be recruited to but also gain access to the damage site, which is normally packed inside a nucleosome. Consequently, specific histone modifications and chromatin remodeling are essential steps of DDR [53].

The histone variant **H2AX** is phosphorylated by ATM (and its kinase family members: ATR and DNA-PK) in response to DNA double strand breaks and is part of the cascade that leads to DNA repair [53]. Two miRNAs have been determined to target H2AX: miR-24 and

miR-138. Overexpressing these two miRNAs leads to higher chromosomal breaks and sensitivity to ionizing radiation and other cytotoxic drugs in various cell lines [54,55].

The chromatin remodeling factor **SNF2H/SMARCA5** is part of the ACF1 complex and is essential for both NHEJ and HR repair pathways [56]. SNF2H is a direct target of the miR-99 family miRNAs: miR-99a and miR-100 [57], and overexpression of either of these miRNAs blocks both DSB repair pathways [35].

Additionally, miR-99 family miRNAs are upregulated upon irradiation in several cancer cell lines [26,35]. As a result, while SNF2H level can promptly increase following the first exposure to IR, radiation-induced upregulation of miR-99a/100 mitigate the rise of SNF2H level in subsequent rounds of irradiation [35]. The practice of dividing the total dose of radiation used in radiotherapy, or *fractionation*, arose empirically [1]. This experiment may help explain the molecular basis of the benefit of fractionation over single-dose treatment by showing that, due to miR-99a/100 and SNF2H, DNA repair is less efficient when cells are repeatedly irradiated [35].

Cell-cycle checkpoints

Cell-cycle checkpoints are charged with the mission of triggering cell-cycle arrest to allow time for DNA repair. Once the damage is repaired, cells will re-enter the cell cycle; or if the DNA damage is too severe, apoptosis or senescence will be the ultimate outcomes [58]. DNA-damage checkpoints include several complex pathways. Two proteins under miRNA-mediated regulation will be discussed here.

One major factor in DNA-damage checkpoint activation is the transcription factor **p53** (reviewed in [58]). p53 is a direct target of several miRNAs, including miR-125b, miR-504, and miR-33 (reviewed in [59]). Conversely, p53 mediates the processing and functions of miRNAs at several steps including transcription induction, processing by DROSHA and DICER, and mRNA target selection via interaction with RNA-binding-motif protein 38 (RBM38) (reviewed in [59]). One example is miR-34 whose transcription is induced by p53 in response to DNA damage [30]. It has been shown that the absence of miR-34 causes cells to be sensitive to non-apoptotic cell death, but protects cells from apoptotic cell death [32]. Another study also confirms that overexpression of miR-34b sensitizes cells to IR, but this effect was not observed in p53-negative cells [39].

The Cdk family includes the key kinases that promote passage through cell-cycle checkpoints. Cdks are phosphorylated, and thus inhibited, after DNA damage, and they are activated again by the Cdc25 family phosphatases to lift the checkpoint arrest. Therefore, Cdc25 is degraded upon irradiation while DNA repair needs to occur [58]. **Cdc25a** is a direct target of miR-21 [60] and *let-7* [61]. Modifying miR-21 level affects G2/M checkpoint arrest in colon cancer and glioma cell lines [40,60]; whereas modulating *let-7* miRNAs levels has been shown to affect cellular radiosensitivity [16,26,37,38].

DNA repair machinery

While the pathways mentioned above need to coordinate for DNA repair to take place, here we describe the actual machinery of DNA repair. There are many repair pathways, depending on the nature of the DNA damage lesions, but the most common damage from IR is DNA double-strand breaks. Homologous recombination (HR) is a DSB repair pathway that preserves the integrity of the genome as it employs the sister chromatid of the damage sequence as a template [2]. One factor involved in HR is **BRCA1**, which is targeted by miR-182 [62]. Overexpressing miR-182 reduces HR repair efficiency and sensitizes cells to IR [62]. Not only does BRCA1 work in the DNA repair pathway, but it has also been shown

to function in transcriptional regulation. BRCA1 epigenetically represses miR-155 expression by interacting with a histone deacetylation protein to affect the miR-155 promoter [63]. Interestingly, miR-155 itself has been shown to influence the radioresponse in lung cancer cell lines [64].

Another DSB repair pathway is nonhomologous end joining (NHEJ) repair. NHEJ, by the nature of its mechanism, could lead to loss of the integrity of the genome as it uses no template [2]. One essential factor in NHEJ is DNA-dependent protein kinase catalytic subunit (**DNA-PKcs**), which is a direct target of miR-101 (also a regulator of ATM as mentioned above) [50]. Overexpression of miR-101 could sensitize cell lines to IR, and a xenograft of miR-101-overexpressing cells in mice yields tumors that shrink more than the control after IR [50]. Yet, ectopic overexpression of miR-101 does not further radiosensitize cells that normally have high level of miR-101 to begin with [65].

Proteins regulating formation of reactive oxygen species

IR leads to production of harmful reactive oxygen species, including superoxide (O_2^-), which causes damage to DNA. The superoxide dismutase (SOD) family of proteins catalyzes the conversion of superoxide to hydrogen peroxide, one step in metabolizing and disposing of it. miR-21 directly targets and downregulates **SOD3** and indirectly decreases SOD2 level via reduced **TNFα** expression, thus leading to increased superoxide levels [66] and possibly, increased DNA damage.

The MAPK pathways

The mitogen-activated protein kinase (MAPK) pathways are a group of signal transduction pathways that link growth factors to multiple processes including proliferation, senescence, differentiation, and apoptosis. Many proteins in this pathway have been shown to play a role in radiation responses, especially in cell survival and subsequent repopulation after IR (reviewed in [14]).

The MAPK pathways initiate with transmembrane receptors, which transduce signal through several small GTPases, including the ERBB receptors' association with **KRAS**. *let-7*, a tumor suppressor miRNA, downregulates many pro-survival genes including *RAS* and *MYC* [11,13], and LIN-28 functions in negatively regulating *let-7* biogenesis [27]. Two studies link the LIN-28-*let-7* axis to radiosensitivity through KRAS [37,38], which has been previously shown to protect cells from IR [14]. Both studies found that overexpressing *let-7a* [37] or *let-7g* [38] sensitizes lung cancer cell lines to ionizing radiation. Knockdown of LIN-28 by siRNA also leads to increased *let-7* levels and higher radiosensitivity [37,38].

Conclusions

In recent years, there have been many reports documenting the change in miRNA expression upon IR from different cell types and of the specific role of various miRNAs on cellular radiosensitivity. For example, *let-7* miRNAs change in expression levels upon irradiation in a wide variety of cells [15–26]. Not only are *let-7* miRNAs under the regulation of a key DNA damage-response gene like p53 [28], but they also influence cell survival through several targets including Cdc25a, KRAS, MYC, and NFκB1 [11,13,16,61]. That is also the case for miR-21 which regulates several pathways that are essential for cell survival after radiation, including ROS metabolism, PTEN, and cell-cycle checkpoints [40,60,66]. Moreover, miR-21 biogenesis is increased after IR by KSRP, which is activated by DNA damage-induced ATM phosphorylation [48].

It is now clear that miRNAs are important players in this complex response to radiation. These data strongly suggest that miRNAs could prove useful in modulating radioresponsitivity at the clinical level, for instance, by modifying *let-7* family miRNAs or miR-21 levels. At present, most miRNAs have been investigated and tested in cell culture systems, and further studies will need to demonstrate the effect of miRNAs on radiosensitivity in *in vivo* models, not unlike those conducted for miRNAs as cancer therapeutics. Concurrently, much progress has been made in miRNA delivery techniques [7]. We speculate that both efforts should culminate in the successful development of a new class of radiosensitizing agents for cancer patients.

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Table 1

miRNAs whose levels of expression are affected by IR in various cell lines

Increase	Decrease	Increase	Decrease
let-7a [22]	let-7a [18,21,26]	miR-34b* [19,20]	
let-7b [22]	let-7b [18,21,26]	miR-34c [31](2 cell types)	
let-7c [20,22]	let-7c [18,26]	miR-99a [26,35]	miR-99a [19]
let-7d [20–22]	let-7d [17,18,26]	miR-100 [35]	miR-100 [19,21,31]
let-7e [17,21,22]	let-7e [18,19,26]	miR-106a [23,26]	miR-106a [15]
let-7f [17,22,23]	let-7f [18,20,26]		miR-107 [24,31]
let-7g [20–25]	let-7g [15,18]		miR-125a [25,26]
let-7i [17,21,22]	let-7i [18,26]	miR-126 [23,34]	
	miR-10a [15,19]		miR-133b [31](2 cell types)
miR-15a [17,22,26]		miR-142-3p [17,22]	
miR-16 [19,22,23,25,26]		miR-142-5p [17,22,23]	
miR-17-3p [15,17,22,23]	miR-17 [19]	miR-143 [17,22]	miR-143 [23,31]
miR-17-5p [15,17,22,23,26,34]		miR-145 [17,19,23]	miR-145 [31]
miR-19a [22,23,26,34]		miR-148a [23,26]	
miR-19b [17,22,26,34]	miR-19b [19]		miR-152 [15,19]
miR-20a [23,25,34]		miR-155 [17,22]	miR-155 [17,26]
miR-20b [23,26]			miR-181a [19,24]
miR-21 [17,21–23,25,34–36]		miR-188-5p [19,20]	
miR-22 [24,26,67]			miR-196a [19,31]
miR-24 [23,26,67]	miR-24 [21]	miR-191 [24,67]	
miR-26b [21,23]	miR-26b [16]	miR-221 [19,23,26,34]	
miR-27a [23,26]		miR-222 [23,34]	miR-222 [21]
miR-27b [15,26,34]		miR-365 [20,26]	
miR-29a [23,34]		miR-379 [24,31]	
miR-29c [23,25,34]			miR-521 [24,31]
miR-30a-5p [26,67]		miR-601 [19,24]	
miR-34a [19,20]		miR-663 [19,21]	

Table 2

miRNAs and factors that affect radiosensitivity

miRNAs	more sensitive to IR	less sensitive to IR	change in expression
AGO2	knockdown of AGO2 [44]		n/a
DICER	knockdown of DICER [44]		n/a
let-7a	pre-let-7a [37]		varied (increase [22], decrease [18,21,26])
let-7g	pre-let-7g [16,25,38]	anti-let-7g [25]	varied (increase [20–25], decrease [15,18])
miR-7	pre-miR-7 [68]	anti-miR-7 [68]	n/a
miR-9	miR-9 overexpression [16]		*decrease [16]
miR-17-92		miR-17-92 cluster overexpression [69]	*increase [15,17,22,23,25,26,34]
miR-18a	miR-18a overexpression [52]	miR-18a inhibitor [52]	varied [17]
miR-21	miR-21 inhibitor [40]		*increase [17,21–23,25,34]
miR-34b	miR-34b overexpression [39]		n/a
miR-100	miR-100 overexpression [51]		*decrease [19,21,31]
miR-101	miR-101 overexpression [50]	miR-101/-101* inhibitors [50]	increase [23]
miR-125a	anti-miR-125a [25]	pre-miR-125a [25]	decrease [25,26]
miR-127	pre-miR-127 [25]	anti-miR-127 [25]	*decrease [25]
miR-138	miR-138 mimic [55]		n/a
miR-155	anti-miR-155 [64]	pre-miR-155 [64]	varied (increase [17,22,64], decrease [17,26])
miR-181a	miR-181a overexpression [24]		*decrease [19,24]
miR-189	anti-miR-189 [25]	pre-miR-189 [25]	decrease [25]
miR-216a	miR-216a inhibitor [44]		*increase [44]
miR-221/222	anti-miR-221/222 [70]	pre-miR-221/222 [70]	*miR-221 increase [19,23,26,34] *miR-222 increase [23,34]
miR-421	miR-421 overexpression [49]		increase [19]
miR-518-5p	miR-518-5p inhibitor [44]		*increase [44]
miR-521	miR-521 mimic [31]	miR-521 inhibitor [31]	*decrease [24,31]
miR-525-3p	miR-525-3p inhibitor [44]		*increase [44]
miR-628-5p	miR-628-5p inhibitor [44]		*increase [44]